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13. ABSTRACT *(Maximum 200 Words)*
In this report, we have investigated the effects of overexpression of active Ras within the mammary epithelium. Using the tetracycline-inducible system, we have generated a line of mice which, when also bearing rtTA transgene expressed under control of the MMTV LTR, inducibly express active H-Ras in the mammary epithelium in response to the drug doxycycline. We have shown that the mammary epithelium undergoes a large amount of proliferation in response to Ras. Removal of doxycycline abrogates the hyperplastic phenotype and restores a normal gland within 60 days. We have also shown that pathways downstream of Ras are activated, such as the Raf/MEK/MAPK pathway. Furthermore, the p19/p53/p21 pathway is also activated in response to Ras.

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FOREWORD

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INTRODUCTION

The small GTPase protein Ras is one of the most commonly mutated genes in human cancers. Over 20% of human cancers harbor activating mutations in one of three Ras family members, H-, K-, or N-Ras(1). Ras is a signal transducer known to be downstream from numerous cell surface receptors, among them molecules implicated in breast cancer such as the EGFR and erbB2 receptor tyrosine kinases(2,3). Stable transfection of Ras into NIH3T3 cells will transform them into malignant sarcomas, and female mice constitutively expressing activated H-Ras under the control of the MMTV promoter develop mammary carcinomas(4,5). Blockade of Ras signaling, in contrast, is inhibitory to cell growth in cultured cell lines and can inhibit tumor growth in vivo(6,7).

In direct contrast to results obtained using immortalized cell lines, retroviral infection of Ras into primary murine embryonic fibroblasts (MEFs) has been shown to induce a short period of proliferation, followed by a growth arrest that shares many features with cellular senescence(8). Moreover, the growth arrest activated by Ras has also been shown to require functional p53 and INK4a/ARF pathways(8). The genetic loci encoding these proteins are the two most commonly mutated loci in human cancers, suggesting the mechanism underlying Ras-induced growth arrest may be an essential tumor suppressive mechanism present within cells.

Normal MEFs cultured *in vitro* arrest growth prior to telomere dysfunction, thought to be due to genotoxic stress arising from hyperoxic culture conditions(9). The senescent-like phenotype caused by Ras overexpression in primary cells also occurs with normal telomere function(10,11). It is therefore fair to question whether Ras-induced senescence in fact represents an innate protective mechanism against the activation of a potent oncogene, or merely the extension of an artifact of *in vitro* culture conditions. To address this requires the overexpression of Ras in somatic tissues *in vivo*, and the determination of whether any senescent response occurs in the cells expressing Ras. We have generated a line of mice capable of inducibly overexpressing activated H-Ras within the mammary epithelium. Using this line of mice we will determine if growth arrest and/or apoptosis occurs in the mammary epithelium in response to activation of Ras. We will also determine if markers of senescence are detectable in Ras-expressing cells. Finally, we will breed MTB/TRAS mice to p53 knockout mice to determine if a p53-dependent growth arrest occurs in response to Ras.

BODY

The goals of my pre-doctoral fellowship "Determination of a Senescent of Response to Oncogenic Ras Mutation *in Vivo*", are to 1) Generate a line of mice inducibly expressing activated Ras within the mammary epithelium, 2) Characterize the proliferative, apoptotic, and senescent responses of mammary epithelial cells to activated H-Ras, and 3) Determine if the phenotype induced by activated H-Ras is dependent on p53.

We have previously generated a line of mice, designated MTB, which express the reverse tetracycline dependent transactivator (rtTA) under control of the mouse mammary tumor virus (MMTV) LTR(12). MTB female mice express rtTA within the mammary epithelium and salivary gland but in no other organs(12). We have also generated a line of mice harboring a transgene that expresses viral activated H-Ras under control of the tetracycline operon, called the TRAS line of mice. In response to the drug doxycycline, bitransgenic MTB/TRAS mice rapidly and inducibly express active H-RAS within the mammary gland at the RNA and protein levels (data not shown). The transcript expressing H-Ras also contains an IRES-Luciferase cassette such that Luciferase expression may be used as a surrogate for transgene expression. Luciferase assays of uninduced and induced mice have verified that uninduced bitransgenic mice express luciferase levels near those of control monotransgenic mice, while induced MTB/TRAS mice have a large induction of luciferase activity (data not shown). Luciferase activity was detectable at such levels only within the mammary and salivary glands (data not shown). We therefore conclude that the MTB/TRAS system represents a useful way to specifically and inducibly express H-Ras within the mammary epithelium.

To verify that the Ras transgene expressed is biologically active, we have examined the levels of activation of pathways known to be downstream of Ras. Perhaps the best characterized pathway that is downstream of Ras is the MAP kinase pathway. Immunoblots for phospho-MEK and phospho-ERK demonstrated that these pathways are activated within 24 hrs of Ras activation by dox. Furthermore, specifically immunoprecipitating the GTP-bound form of Ras, which is the active form of Ras, demonstrates that while total levels of Ras increase only several fold in response to dox, the levels of GTP-bound Ras increase dramatically in response to dox.

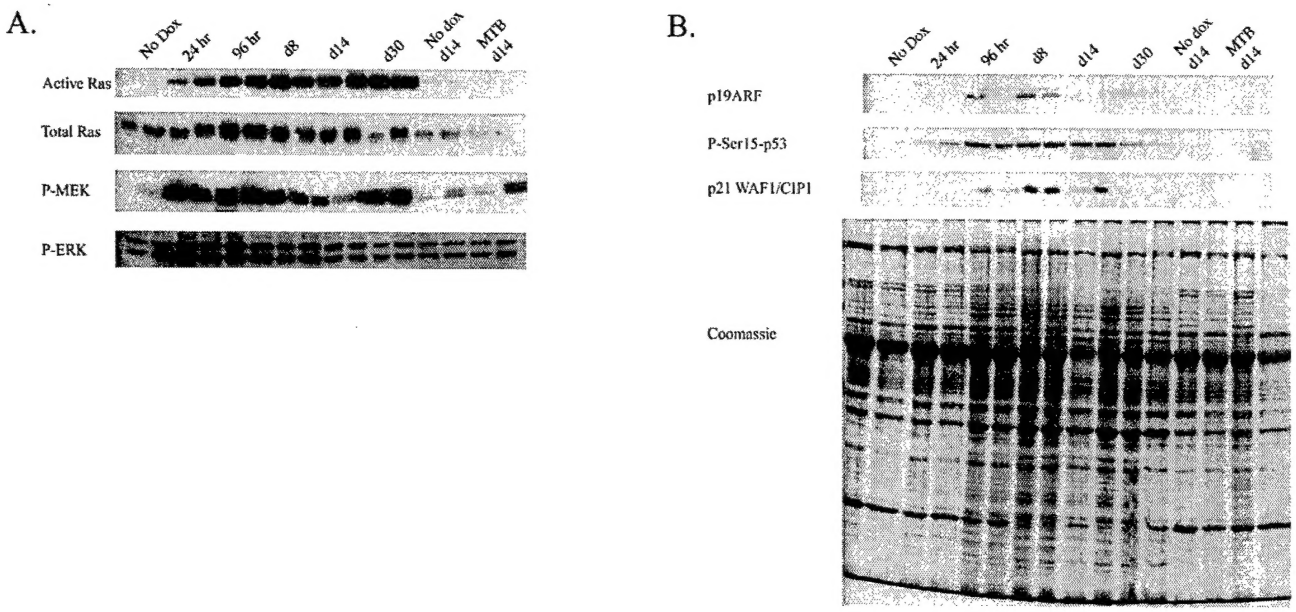

Figure 1 – MTB/TRAS mammary gland protein extracts from mice induced with 0.05 mg/mL dox for varying amounts of time were immnuoblotted with antibodies to the indicated proteins from the Ras/MAPK pathway (A), or p53 pathway (B).

MTB/TRAS bitransgenic mice experience a dramatic mammary epithelial growth in response to induction of Ras. Carmine stained whole mounts of mammary glands induced with doxycycline show that mammary epithelium initially has a large burst of proliferation during the first 4-8 days of induction, followed by a phase of contraction of the mammary epithelium into a spheroid structure from approximately 8-30 days. We will determine if a senescent growth arrest is occurring within the mammary gland during this phase of

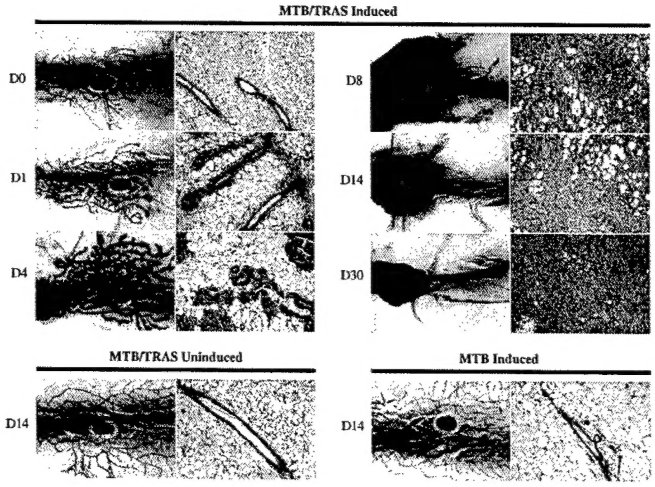

Figure 2 – Whole mounts of inguinal mammary glands from MTB/TRAS mice induced for the indicated times with 0.05 mg/mL dox were stained with carmine (left panels) or sectioned and stained with hemotoxylin and eosin (right panels)

induction. To determine whether there is a senescent or apoptotic response to the Ras in these cells, we are performing BrdU and TUNEL analysis on mammary gland sections from 24 hr, 96 hr, d14, and d30 of dox induction to define the proliferative and apoptotic events, respectively, that occur following transgene induction.

One of the hallmarks of Ras-induced senescence in fibroblasts is that activation of the p53 tumor suppressor occurs due to induction p19ARF, an inhibitor of Mdm2, which in turn acts as an ubiquitin ligase for p53. We wished to determine if these proteins were activated in response to H-Ras. Immunoblots for p19, p53 phospho-serine 15, and the p53 target gene p21WAF1/CIP1 show that the p53 pathway is indeed activated in the mammary epithelium, with maximal activation of the pathway occurring 8-14 days post-induction with dox. We will also look for additional markers of senescence in mammary glands from induced MTB/TRAS mice, including increased PAI-1 RNA levels and increased senescence-associated β-galactosidase activity.

Finally, we will determine if p53 regulates Ras-induced growth arrest *in vivo*. Senescence is thought to be a p53-dependent protective mechanism against tumorigenesis, by arresting the growth of cells containing oncogenic mutations. We will breed MTB/TRAS mice to p53 null mice, and generate MTB/TRAS/p53-/- mice. We will determine the levels of proliferation and apoptosis in these mice by performing BrdU and TUNEL analyses on uninduced, d4, and d14 induced mammary tissue. MTB/TRAS/p53-/- mammary glands when induced with doxycycline would be predicted to have an increased growth rate compared to MTB/TRAS/p53+/+ glands upon induction with Ras, as senescence has been shown in fibroblasts to be a p53-dependent process and p53 null MEFs become transformed upon retroviral infection with active Ras. Mammary glands from MTB/TRAS/p53-/- and MTB/TRAS/p53+/+ mice will be transplanted onto syngeneic wild type hosts for induction, if MTB/TRAS/p53-/- mice develop non-mammary tumors too quickly to monitor mammary epithelial cell growth rates in response to Ras.

STATEMENT OF WORK

Specific Aim 1. Generate a line of mice inducibly expressing activated Ras within the mammary epithelium: Months 12-24

1. Generate bitransgenic MTB/TRAS mice and verify tissue-specific response to doxycycline: Months 12-18
2. Determine doxycycline dose responsiveness of MTB/TRAS mice: Months 18-24
3. Verify that the MAP kinase pathway is activated in response to transgenic Ras: Months 18-24

Specific Aim 2. Characterize the proliferative, apoptotic, and senescent responses of mammary epithelial cells to activated H-Ras: Months 24-30

1. Characterize proliferative and apopotic rates in uninduced and induced MTB/TRAS mammary glands: Months 24-30
2. Determine activation of p53 and p19 pathways in uninduced and induced MTB/TRAS mammary glands: Months 24-30
3. Determine levels of PAI-1 and senescence-associated β-galactosidase activity in uninduced and induced MTB/TRAS mammary glands: Months 24-30

Specific Aim 3. Determine if the phenotype induced by activated v-H-Ras is dependent on p53: Months 30-36

1. Generate MTB/TRAS/p53-/- and MTB/TRAS/p53+/+ female mice: Months 30-36
2. Induce above mice for 0, 4, and 14 days, and compare carmine stained whole mounts and H & E sections for gross differences in morphology: Months 30-36
3. Determine proliferation and apoptotic rates in induced MTB/TRAS/p53-/- and MTB/TRAS/p53+/+ mammary glands: Months 30-36

KEY RESEARCH ACCOMPLISHMENTS

- MTB/TRAS female mice inducibly express active H-Ras within the mammary and salivary glands
- The MAP kinase pathway is activated in response to Ras in mammary glands from MTB/TRAS mice
- Mammary glands from MTB/TRAS mice induced to express H-Ras develop dramatic increases in epithelial cell number based on gross morphology and histologic stains
- The p19/p53 pathway is activated in response to Ras in mammary glands from MTB/TRAS mice

REPORTABLE OUTCOMES

CONCLUSIONS

We have generated a bitransgenic system whereby the effects of activation of a potent oncogene, v-H-Ras, within the adult mammary epithelium may be determined. MTB/TRAS mice specifically and inducibly express activated H-Ras within mammary epithelia of MTB/TRAS mice, but not in uninduced bitransgenics or monotransgenic mice. Furthermore, we have demonstrated the the Ras transgene expressed is biologically active, in that the MAP kinase pathway is activated in response to Ras induction. In addition to the MAP kinase pathway, we have also shown that the p53 pathway is activated in response to this oncogene, as also occurs in MEFs in vitro. We have demonstrated that there is an initial response of epithelial proliferation in response to Ras, although the chronic effects of Eas expression in the mammary epithelium remain undetermined.

Our future experiments will be to further characterize the phenotype of MTB/TRAS mammary glands exposed to H-Ras. We will examine additional markers for senescence in these mice, an increase in PAI-1 RNA and in senescent-associated β-galactosidase activity(13,14). We will more precisely define the proliferative and apoptotic status of the mammary epithelium in response to Ras by performing BrdU and TUNEL analyses on MTB/TRAS glands induced with dox for varying amounts of time. Finally, we will determine if loss of p53 renders MTB/TRAS mice increasingly susceptible to mammary carcinogenesis by generating MTB/TRAS/p53-/- mice and analyzing the mammary glands for transformation in response to induction with Ras.

REFERENCES

1. Bos, J. L. (1989) *Cancer Res* **49**, 4682-9
2. Deuel, T. F. (1987) *Annual Review of Cell Biology* **3**, 443-92
3. Galang, C. K., Garcia-Ramirez, J., Solski, P. A., Westwick, J. K., Der, C. J., Neznanov, N. N., and Oshima, R. G. H., C.A. (1996) *Journal of Biological Chemistry* **271**, 7992-8
4. McCoy, M. S., Toole, J. J., Cunningham, J. M., Chang, E. H., Lowy, D. R., and Weinberg, R. A. (1983) *Nature* **302**, 79-81
5. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. (1987) *Cell* **49**, 465-75
6. Sun, J., Qian, Y., Hamilton, A. D., and Sebti, S. M. (1995) *Cancer Research* **55**, 4243-7
7. Tokumitsu, Y., Nakano, S., Ueno, H., and Niho, Y. (2000) *Journal of Cellular Physiology* **183**, 221-7
8. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) *Cell* **88**, 593-602
9. Sherr, C. J., and DePinho, R. A. (2000) *Cell* **102**, 407-10
10. Jones, C. J., Kipling, D., Morris, M., Hepburn, P., Skinner, J., Bounacer, A., Wyllie, F. S., Ivan, M., Bartek, J., Wynford-Thomas, D., and Bond, J. A. (2000) *Molecular & Cellular Biology* **20**, 5690-9
11. Wei, S., Wei, S., and Sedivy, J. M. (1999) *Cancer Research* **59**, 1539-43
12. Gunther, E. J., Belka, G. K., G.B., W., Wang, J., Hartman, J. L., Boxer, R. B., and Chodosh, L. A. (2002) *FASEB Journal* **16**, 283-92
13. Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S. W. (2000) *Genes & Development* **14**, 2015-27
14. Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. (2000) *Nature* **406**, 207-10